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Effects of Bacterial Community Members on the Proteome of the Ammonia-Oxidizing Bacterium *Nitrosomonas* sp. Strain Is79

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ABSTRACT

Microorganisms in the environment do not exist as the often-studied pure cultures but as members of complex microbial communities. Characterizing the interactions within microbial communities is essential to understand their function in both natural and engineered environments. In this study, we investigated how the presence of a nitrite-oxidizing bacterium (NOB) and heterotrophic bacteria affect the growth and proteome of the chemolithoautotrophic ammonia-oxidizing bacterium (AOB) *Nitrosomonas* sp. strain Is79. We investigated *Nitrosomonas* sp. Is79 in co-culture with *Nitrobacter winogradskyi*, in co-cultures with selected heterotrophic bacteria, and as a member of the nitrifying enrichment culture G5-7. In batch culture, *N. winogradskyi* and heterotrophic bacteria had positive effects on the growth of *Nitrosomonas* sp. Is79. An isobaric tag for relative and absolute quantification (iTRAQ) liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics approach was used to investigate the effect of *N. winogradskyi* and the co-cultured heterotrophic bacteria from G5-7 on the proteome of *Nitrosomonas* sp. Is79. In co-culture with *N. winogradskyi*, several *Nitrosomonas* sp. Is79 oxidative stress response proteins changed in abundance, with periplasmic proteins increasing and cytoplasmic proteins decreasing in abundance. In the presence of heterotrophic bacteria, the abundance of proteins directly related to the ammonia oxidation pathway increased, while the abundance of proteins related to amino acid synthesis and metabolism decreased. In summary, the proteome of *Nitrosomonas* sp. Is79 was differentially influenced by the presence of either *N. winogradskyi* or heterotrophic bacteria. Together, *N. winogradskyi* and heterotrophic bacteria reduced the oxidative stress for *Nitrosomonas* sp. Is79, which resulted in more efficient metabolism.

IMPORTANCE

Aerobic ammonia-oxidizing microorganisms play an important role in the global nitrogen cycle, converting ammonia to nitrite. In their natural environment, they coexist and interact with nitrite oxidizers, which convert nitrite to nitrate, and with heterotrophic microorganisms. The presence of nitrite oxidizers and heterotrophic bacteria has a positive influence on the growth of the ammonia oxidizers. Here, we present a study investigating the effect of nitrite oxidizers and heterotrophic bacteria on the proteome of a selected ammonia oxidizer in a defined culture to elucidate how these two groups improve the performance of the ammonia oxidizer. The results show that the presence of a nitrite oxidizer and heterotrophic bacteria reduced the stress for the ammonia oxidizer and resulted in more efficient energy generation. This study contributes to our understanding of microbe-microbe interactions, in particular between ammonia oxidizers and their neighboring microbial community.

Nitrification, the oxidation of ammonia (NH_3) to nitrate (NO_3^-) via nitrite (NO_2^-), is a microbially driven two-step process within the global nitrogen cycle (1, 2). The first step, the oxidation of NH_3 to NO_2^-, is carried out aerobically by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) (3–6). AOB are chemolithoautotrophic bacteria that generate energy through ammonia oxidation and fix carbon through the Calvin-Benson-Bassham cycle (3). The second step of nitrification is performed by nitrite-oxidizing bacteria (NOB), which generate energy from the oxidation of NO_2^- to NO_3^- (7–9). Recently, it has been discovered that members of the phylum *Nitrospira* are able to perform complete ammonia oxidation (i.e., comammox), oxidizing NH_3 via NO_2^- to NO_3^- (10, 11).

Betaproteobacterial AOB are comprised of the genera *Nitrosomonas* and *Nitrosospira*. Within the genus *Nitrosomonas*, AOB form several clusters that can be distinguished by ecophysiological characteristics, such as affinity for NH_4^+ and tolerance to increasing salt or NO_2^- concentrations (1, 6, 12). *Nitrosomonas* cluster 6a is comprised of AOB adapted to low ammonium (NH_4^+) concen-

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trations that can be found in a range of environments (13–17). *Nitrosomonas* sp. strain Is79, a member of *Nitrosomonas* cluster 6a, was isolated from the freshwater sediment nitrifying enrichment culture G5-7 (18–20).

In the environment, microorganisms do not exist in isolation but live as members of complex communities of interacting microbial species. Within microbial communities, negative interactions such as competition or predation can be detected along with mutually beneficial or synergistic interactions such as cross-feeding, co-metabolism or cell-to-cell communication (21–23). Photo- and chemolithoautotrophic microbes interact with heterotrophic microbes in many different environments (24, 25). For example, macro- and microalgae live with specific heterotrophic microbial subcommunities. The exchange of nutrients, enzymes, and bioactive compounds in these communities results in increased community productivity (24, 25). AOB have been shown to interact positively with NOB and heterotrophic bacteria (26–30). The positive effect of NOB on the growth of AOB is in large part due to the removal of NO_2^- by NOB, as it inhibits AOB growth (8, 29, 31). In the transcriptome of *Nitrosomonas europaea*, increases in transcript abundance for electron transport proteins (NADH dehydrogenase complex, ATP synthase complex, and several cytochromes, including cytochrome c_{552}) were identified as possible positive effects of co-cultivation with the NOB *Nitrobacter winogradskyi* (29). In addition, AOB provide organic carbon in the form of soluble microbial products to co-cultured heterotrophic bacteria (26, 27, 32). The positive growth response of AOB to the presence of heterotrophic bacteria could be due to a variety of compounds that may be secreted by the heterotrophic bacteria, such as organic compounds (33–36), acyl-homoserine lactones (37, 38), or siderophores (28). Studies of AOB-heterotroph interactions have mainly focused on the members, structure, and functionality of the heterotrophic bacterial communities (26, 27). It has not been elucidated how the proteomes of AOB respond to the presence of co-cultured NOB or heterotrophic bacteria.

To our knowledge, only a few studies have been conducted focusing on the transcriptome or proteome of AOB. These studies have mainly utilized *Nitrosomonas europaea* or *Nitrosomonas eutropha*, two relatively fast-growing AOB often found in nutrient-rich environments (29, 39–43). The AOB proteome analyses have focused on the effect of variable environmental or physiologic growth conditions, such as starvation (42), heavy-metal contamination (41), biofilm formation (43), and the effect of nitrogen dioxide (NO_2) or anoxia (40).

Here, we present a study investigating the effects of *N. winogradskyi* as well as co-cultured heterotrophic bacteria isolated from the enrichment culture G5-7 on the growth and physiology of the AOB *Nitrosomonas* sp. Is79. We employed a combination of growth experiments and isobaric tag for relative and absolute quantification (iTRAQ) liquid chromatography-tandem mass spectrometry (LC-MS/MS) proteomics to determine how the proteome of *Nitrosomonas* sp. Is79 is influenced by the presence of *N. winogradskyi* and heterotrophic bacteria.

MATERIALS AND METHODS

Cultures and media. (i) Cultures. We utilized the following cultures derived from the original AOB enrichment culture G5-7 (18, 19): (i) AOB isolate *Nitrosomonas* sp. Is79 (20), (ii) the AOB enrichment culture G5-7 itself, which contained *Nitrosomonas* sp. Is79, *N. winogradskyi*, and co-cultured heterotrophic bacteria, and (iii) heterotrophic bacteria isolated from the enrichment culture G5-7. G5-7 was originally enriched in a chemostat with an initial NH_4^+ concentration of 0.05 mM using *Glyceria maxima* rootzone sediment (18). The chemostat culture was transferred to batch culture using an initial NH_4^+ concentration of 0.25 mM first and 5 mM later. The enrichment culture was transferred every 4 to 6 weeks to fresh medium. *Nitrosomonas* sp. Is79 was isolated from the enrichment culture G5-7 using serial dilution, and the complete genome has been sequenced (20). The NOB *N. winogradskyi* (ATCC 25391) was also used in the experiments.

(ii) Medium to cultivate AOB and NOB. The AOB mineral salts (AOB-MS) medium used for AOB growth experiments contained various amounts of $(NH_4)_2SO_4$, 10 mM NaCl, 1 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.2 mM $MgSO_4 \cdot 7H_2O$, and 1 ml · liter^{-1} trace element solution (44, 45). Batch cultures were buffered with HEPES, which was added in a 4-fold molar ratio to the NH_4^+ concentration, and the pH was adjusted to 7.8 before autoclaving (44). After autoclaving, sterile KH_2PO_4 was added to a final concentration of 0.4 mM. *N. winogradskyi* was cultivated in NOB mineral salts (NOB-MS) medium that contained 14.5 mM $NaNO_2$, 8.6 mM NaCl, 1.1 mM KH_2PO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 0.03 mM $CaCO_3$, and 1 ml · liter^{-1} trace element solution with a pH of 7.8 (45, 46).

(iii) Media to cultivate the heterotrophic bacteria. LB medium (Sigma-Aldrich, St. Louis, MO, USA), R2A medium (47), and spent AOB-MS medium were used to isolate and cultivate heterotrophic bacteria. Spent AOB-MS medium consisted of 25% sterile filtered medium from an outgrown *Nitrosomonas* sp. Is79 culture, 75% AOB-MS medium, and either 0.01 g/liter Casamino Acids (Difco, Becton, Dickinson and Company, Sparks, MD, USA) or 0.01 g/liter yeast extract (Difco). If necessary, medium was solidified with 1.5% Bacto agar (Difco).

(iv) Isolation of heterotrophic bacteria from the enrichment culture G5-7. Late log phase G5-7 culture was serially diluted with AOB-MS medium without NH_4^+ and inoculated onto agar plates containing either LB, 0.1× LB, R2A, or spent AOB-MS medium. Individual colonies were selected based on colony morphology, subcultured, and further maintained on R2A agar plates. Colony PCR and 16S rRNA gene sequencing were used to identify the isolated colonies.

(v) Contamination tests. Axenic cultures of *Nitrosomonas* sp. Is79 and *N. winogradskyi* were inoculated into LB medium (0.1×) and incubated at 27°C for 2 weeks to test for purity. Cultures exhibiting growth of heterotrophic bacteria were discarded.

Kinetic studies. (i) Apparent Michaelis constant. Batch cultures of *Nitrosomonas* sp. Is79 grown as a pure culture, in co-culture with *N. winogradskyi* and in the enrichment culture G5-7 were cultivated with continuous stirring and bubbling at 25°C in the dark. Late logarithmic growth phase cells (1 liter) were harvested by centrifugation (20 min, 30,000 × *g*, 4°C), washed, and resuspended in NH_4^+-free AOB-MS medium (10 ml). NH_4^+-free AOB-MS medium (3.6 ml) was mixed with the concentrated cell suspension (0.4 ml). Concentrated NH_4^+ solution was added to obtain final NH_4^+ concentrations between 20 and 1,000 μM. NO_2^-/NO_3^- production was followed by a NO_2^-/NO_3^- biosensor and used to calculate NO_2^-/NO_3^- production rates (48). Michaelis-Menten kinetics were applied to calculate the apparent half saturation constant of ammonia oxidation [$K_{m(app)}$] (49).

Growth experiments. (i) Half-saturation constant of growth (K_s). The growth rate of *Nitrosomonas* sp. Is79 as a pure culture, in co-culture with *N. winogradskyi* and in the enrichment culture G5-7 was determined across a range of initial NH_4^+ concentrations (0.25 to 5 mM). The cultures were inoculated with 10% (vol/vol) of a late logarithmic phase culture, incubated at 25°C in the dark, and shaken at 120 rpm. Samples were taken regularly to determine NH_4^+, NO_2^- and NO_3^- concentrations. Growth rates were determined by calculating the slope of the log-transformed NO_2^- or NO_2^-/NO_3^- concentrations against time, assuming a correlation between NO_2^-/NO_3^- production and growth (44, 50). Monod kinetics were applied to determine the half saturation constant of growth (K_s) of *Nitrosomonas* sp. Is79 in the different cultures (49).

(ii) Growth experiments of *Nitrosomonas* sp. Is79 with heterotrophic bacteria and *N. winogradskyi*. All growth experiments were conducted in AOB-MS medium with 1 mM NH_4^+. Cultures were inoculated with 1% (vol/vol) late logarithmic phase *Nitrosomonas* sp. Is79 and one or more of the following: 0.2% (vol/vol) R2A medium, 0.2% (vol/vol) culture of heterotrophic bacteria cultivated in R2A, or 1% (vol/vol) *N. winogradskyi* culture. In addition, we used the enrichment culture G5-7. Heterotrophic bacterial cultures were precultured in liquid R2A for 2 days prior to inoculation. All 10 heterotrophic isolates were combined into a mixed culture, which was added to *Nitrosomonas* sp. Is79 to resemble as well as possible the original G5-7 heterotrophic population. When the cultures had consumed the NH_4^+, they were transferred to fresh AOB-MS medium (1% vol/vol). This transfer was repeated once more, resulting in three successive growth cycles. Samples were taken regularly during the third growth cycle, stored at −20°C, and later used to determine NO_2^-/NO_3^- concentrations. Following the third growth cycle, co-cultures were plated on R2A agar. Colony PCR and sequencing were used to confirm the identity of the heterotrophic bacteria.

(iii) Chemostat setup. Chemostats with a working volume of 5 liters were assembled and autoclaved with 4.5 liters of unbuffered AOB-MS medium with 5 mM NH_4^+. Once the temperature was adjusted to 27°C with a temperature blanket, continuous stirring (300 rpm) and bubbling (500 ml · min^{-1}) with 0.2-μm sterile atmospheric air were started. Sterile KH_2PO_4 was added to a final concentration of 0.4 mM and the pH was adjusted to 7.8 with sterile 3% (wt/vol) $NaCO_3$. Chemostats were incubated in the dark and inoculated with late logarithmic phase batch cultures (0.5 liter) of *Nitrosomonas* sp. Is79, *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi*, or the enrichment culture G5-7. Samples (20 ml) were taken at regular intervals, filtered through a polycarbonate filter (0.2 μm, Whatman Nuclepore), and stored at −20°C for chemical analysis. Contamination tests were conducted each time a sample was taken. When the initial NH_4^+ in the chemostat was consumed, unbuffered AOB-MS medium containing 5 mM NH_4^+ was added at a dilution rate of approximately 1.25 liter · day^{-1} (growth rate, 0.25 day^{-1}). Chemostats were run for 12 days, which corresponded to 3 volume changes (equal to 3 generations). Total cell biomass was harvested by centrifugation (20 min, 22,000 × *g*, 4°C). The cell pellets were washed in phosphate buffer (20 mM KH_2PO_4, pH 8), centrifuged (20 min, 28,000 × *g*, 4°C), and stored at −80°C for proteomic analysis.

(iv) Chemical analysis. Colorimetric assays were used to determine NH_4^+, NO_2^-, and NO_3^- concentrations in cell-free supernatants (44, 51–53).

Molecular analysis. (i) Identification of the heterotrophic isolates. Cell material of the isolated heterotrophic bacteria was used as the template for 16S rRNA PCR with the eubacterial primers 27F and 1492R (see Table S1 in the supplemental material) and GoTaq Green master mix (Promega, Madison, WI) (54). PCR products were purified using the Wizard SV gel and PCR cleanup system (Promega, Madison, WI) and sequenced with the two different sequencing primers 357F and 518R (see Table S1) (55) using a BigDye terminator cycle sequencing kit on an Applied Biosystems 3730 DNA analyzer (Life Technology Corporation, Carlsbad, CA, USA) at the Center for Bioinformatics and Functional Genomics (CBFG) at Miami University. The sequences were edited in 4Peaks (A. Griekspoor and T. Groothuis, the Netherlands Cancer Institute). The phylogenetic program ARB was used to align the sequences and to determine the closest cultured relatives (56).

(ii) Molecular analysis of the heterotrophic community in the enrichment culture G5-7 using next-generation sequencing (NGS). The enrichment culture G5-7 (50 ml) was filtered onto a 0.2-μm nucleopore filter (Whatman Nuclepore). DNA was extracted with the FastDNA spin kit for soil (MP Biomedicals, Solon, OH) according to the manufacturer's recommendations, with the following modifications. Filters were homogenized using a bead beater (Biospec Products, Bartlett, OK) three times for 30 s at 4,800 rpm. Samples were stored on ice for 10 min between bead-beating steps and centrifuged (15 min, 28,000 × *g*, 4°C).

Enrichment culture G5-7 DNA was amplified in triplicate with Illumina-compatible indexed primers designed to amplify the V4 region of the 16S rRNA gene (515F-806R) (57). Since the sample was part of a larger sequencing project, a 12-base barcode was incorporated in the reverse primers (57, 58). The PCR products were quantified using a Sybr green I double-stranded DNA (dsDNA) assay, and the concentration was measured on a NanoDrop 3300 fluorospectrometer (Thermo Fisher Scientific, Wilmington, DE, USA). All PCR products were mixed in equal ratio and sequenced in a MiSeq (Illumina, San Diego, CA, USA) at the CBFG at Miami University.

The sequences were processed by the software package MiSeq Reporter into two files: sequences and barcodes. The sequences were split based on the barcodes, quality filtered with a minimum quality score of 25, truncated to 150 bp, and exported as sequence files (fasta) using the software package QIIME (59). The operational taxonomic units (OTUs) were picked against the Greengenes database gg_13_8_otus with 97% similarity. Since there might be an increased diversity in pyrosequencing libraries due to sequencing errors, and to focus on the dominant sequences, all OTUs with abundances below 10 were discarded. The taxonomic affiliations of the sequences were determined, and representative sequences of each OTU were aligned to the ARB database to determine the similarity to the isolated heterotrophic bacteria. The analysis of the community focused on the heterotrophic bacteria.

Protein quantification and analysis. (i) Total cell protein extraction. Wet cell pellets (0.033 to 0.08 g) were suspended in phosphate buffer (500 μl, 20 mM KH_2PO_4, pH 8.0), combined with 1 g of 0.1-mm zirconia/silica beads and bead beaten three times for 30 s at 4,800 rpm. Samples were stored on ice for 10 min between each round. Samples were centrifuged (20 min, 28,000 × *g*, 4°C) and the supernatant was stored at −80°C.

(ii) Solubilization and isolation of peptides. Cell lysates from *Nitrosomonas* sp. Is79, *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi*, and the enrichment culture G5-7 were solubilized in Laemmli gel buffer with heating to 110°C for 10 min. Then, 50 μg of each sample (5 samples total) was loaded onto separate lanes of two one-dimensional, 4% to 12% Bis-Tris acrylamide 1.0-mm mini gels, and electrophoresed for 15 min. The gel region containing the proteins (~1.5 cm by 2.5 cm) was cut from the gel and subjected to in-gel trypsin digestion, and the peptides were recovered as described previously (60).

(iii) iTRAQ labeling. The *Nitrosomonas* sp. Is79 sample was used as the control versus each of the treatments. A total of 25 μg of each sample for the two comparative groups was tagged using iTRAQ reagents following the vendor's (Sciex) instructions and as described previously (61). The 116 and 117 reporter tags were used for the isolated peptides of *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi* samples (Run1) and the enrichment culture G5-7 (Run2), while the 115 reporter tag was used for the *Nitrosomonas* sp. Is79 control sample in both runs. In each case, labeling reagent sufficient to uniformly tag up to 100 μg of digested protein was used, thus ensuring complete labeling of all peptides when using only 25 μg of protein in each labeling reaction. After labeling, samples were mixed in equal quantities for subsequent separation, identification, and quantitative analysis.

(iv) NanoLC-ESI-MS/MS. Nanoscale LC-electrospray ionization-MS/MS (nanoLC-ESI-MS/MS) analyses were performed on a TripleTOF 5600+ (Sciex, Toronto, ON, Canada) coupled to an Eksigent (Dublin, CA) nanoLC.ultra nanoflow system. Then, 2 μg of total protein from each mixture was loaded and analyzed as described by Huang et al. in 2015 (62), with the following modifications. The time for desalting and concentration was increased to 15 min, and 2,916 cycles of time of flight (TOF) MS scans were performed in 80 min. The data were recorded using Analyst-TF (v.1.6) software.

Data analyses. Searches from the nanoLC-MS/MS were accomplished using ProteinPilot software (version 4.5, revision 1656) that utilizes a Paragon algorithm (version 4.5.0.0, revision 1654) against a database consisting of *Nitrosomonas* sp. Is79 and *N. winogradskyi* protein sequences retrieved from the NCBI whole-genome database supplemented with

TABLE 1 Phylogenetic affiliation and abundance of the cultivated bacterial isolates from enrichment culture G5-7 in the 16S rRNA NGS library[a]

Isolate	Closest cultured relative	Similarity (%)	Sequence length (bp)	Abundance (%) in G5-7
Alphaproteobacteria				
Is42	*Afipia broomeae* (U87759)	99.7	1,099	44.8
Is3	*Ancylobacter aquaticus* (M62790)	99.0	625	8.7
Is2	*Afipia* genospecies 7 (U87773)	99.4	1,089	4.4 (= Is28)[b]
Is28	*Mesorhizobium huakii* (D12797)	99.8	1,372	4.4 (= Is2)[b]
Is32	*Caulobacter fusiformis* (AJ007803)	98.5	1,082	1.3
Is17	*Rhizobium radiobacter* (M11223)	96.6	1,071	1.1
Is23	*Sphingomonas adhaesiva* (D13722)	98.8	1,100	0.5
Betaproteobacteria, Is22	*Pandoraea pnomenusa* (AF139174)	99.8	1,076	2.3
Gammaproteobacteria				
Is19	*Pseudomonas putida* (AF094743)	99.6	1,137	23.9
Is39	*Lysobacter antibioticus* (AB019582)	95.7	1,091	2.3

[a] Percent abundance in enrichment culture G5-7 is based on the total reads assigned to OTUs representing heterotrophic bacteria.
[b] Based on the short read length in NGS, Is2 and Is28 cluster together as OTU1 (see Table S2 in the supplemental material).

common contaminating proteins (human keratins, porcine trypsin, etc.) for a total of 12,988 proteins searched. ProteinPilot search parameters included iTRAQ tagging of all primary amines as a variable modification, all biological modifications as variable modifications and carboxyamidomethyl cysteine used as a fixed modification as a result of the reduction and alkylation of any disulfide bond with iodoacetamide prior to trypsin digestion. Gel-based identification (ID) was selected as a special factor in the software to account for any modifications associated with proteins that have been exposed to polyacrylamide gels. The precursor mass tolerance was set to quadrupole-tof instrument in the ProteinPilot software. Data normalization across all iTRAQ tag channels was accomplished in the ProteinPilot software using the bias correction function. This function evaluates the relative ratio of the iTRAQ tags across all the peptides identified and normalizes the ratios, such that the collective ratio of the tags used is 1:1:1 (i.e., 115:116:117 tag), prior to calculating relative differences among individual peptides/proteins. The output files from the ProteinPilot database search contained a summary statistics page, the peptide identification tables, protein identification tables, and relative quantitation data from the iTRAQ reporter ions from each peptide. Further statistical analysis was conducted using the ProteinPilot descriptive statistics template (PDST, version 3.005pB) (http://www.absciex.com/Documents/Downloads/Literature/ProteinPilot-Descriptive-Stats-Template-MassSpec-1910211-01.pdf). This analysis includes processing the relative quantitation data among the sample sets and provides statistical probabilities related to the confidence of the protein identification in relationship to an inverse (decoy) protein database, which consisted of hypothetical proteins based on the inverse amino acid primary sequence of all 12,988 proteins in the database. Only those peptides at 99% or greater confidence (<1% false discovery rate) were presented. *P* values for the quantitative difference between proteins were calculated from the collective reporter ion ratios of all the peptides identified and quantified for a given protein with a minimum of 2 peptide measurements per protein. A significant change in relative protein abundance between the treatment and control was only presented if the abundance of at least 2 peptides of the same protein changed by a factor of at least 1.5 with a *P* value of <0.01 (see Table 3).

Statistical analysis. Statistical analysis (one-way analysis of variance [ANOVA]) was conducted with SSPS (version 19).

Accession number(s). The sequences of the heterotrophic community of the enrichment culture G5-7 determined by NGS were deposited in the NCBI SRA database under BioProject accession no. PRJNA289396. The individual 16S rRNA gene sequences of the isolates were deposited in GenBank under the accession numbers KT267186 to KT267195.

RESULTS

Phylogenetic analysis of the heterotrophic bacteria in the enrichment culture G5-7. Next-generation sequencing (NGS) of the heterotrophic subcommunity of the enrichment culture G5-7 revealed a total of 19 unique OTUs (see Table S2 in the supplemental material). Based on reads, the community was comprised of *Alphaproteobacteria* (70.3%), *Gammaproteobacteria* (24.4%), *Betaproteobacteria* (2.5%), and *Bacteriodetes* (2.8%). Ten unique heterotrophic *Proteobacteria* were isolated from the enrichment culture G5-7 (Table 1). All 10 isolates were detected in the NGS library and comprised 87.7% of the nonnitrifyer sequences (Table 1; see also Table S2 in the supplemental material).

Ammonia-oxidizing activity and growth characteristics of *Nitrosomonas* sp. Is79. *Nitrosomonas* sp. Is79 exhibited a longer lag phase and a lower rate of NO_2^- production in pure culture than when grown in co-culture with *N. winogradskyi* or as part of the enrichment culture G5-7 (Fig. 1). The growth rate of *Nitro-*

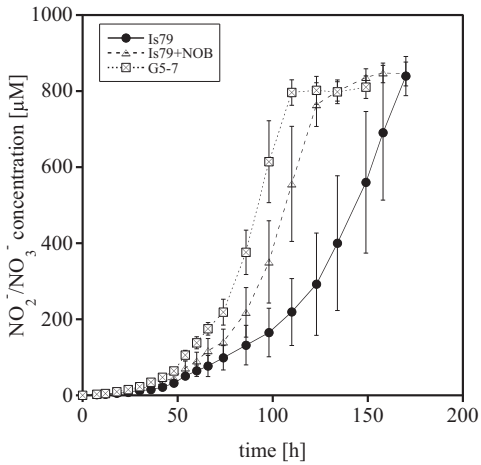

FIG 1 Nitrite or nitrite/nitrate production (μM) by *Nitrosomonas* sp. Is79 (Is79), by *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi* (Is79 + NOB), and by the enrichment culture G5-7 (mean ± standard deviation; $n = 3$).

TABLE 2 Half saturation constant of ammonia-oxidizing activity [$K_{m(app)}$], growth (K_s), and growth rate at 1 mM ammonium of Is79, Is79 + NOB, and G5-7[a]

Measure	Culture		
	Is79	Is79 + NOB	G5-7
$K_{m(app)}$ (μM NH_3)[b]	3.32/3.56	3.10/3.56	3.37/3.53
K_s (μM NH_3)[c]	17.9 ± 3.1 A	9.4 ± 1.9 B	8.2 ± 2.9 B
Growth rate (h^{-1})[c,d]	0.023 ± 0.003 A	0.037 ± 0.002 B	0.055 ± 0.002 C

[a] Is79, *Nitrosomonas* sp. Is79 in pure culture (Is79); Is79 + NOB, Is79 co-cultured with *N. winogradskyi*; G5-7, Is79 cultured as part of the enrichment culture G5-7.
[b] Measurement of biological replicates.
[c] Mean ± SD; $n = 3$. Different capital letters indicate significant differences determined by one-way ANOVA followed by Tukey test ($P < 0.05$).
[d] Growth rate calculated based on the nitrite/nitrate production over time (see Fig. 1).

somonas sp. Is79 significantly increased when co-cultured with *N. winogradskyi* and increased even more when grown as part of the enrichment culture G5-7 (Table 2). The half saturation constant of growth (K_s) decreased significantly when *Nitrosomonas* sp. Is79 was co-cultured with *N. winogradskyi* or grown as part of the enrichment culture G5-7 (Table 2). However, the apparent Michaelis constant [$K_{m(app)}$] of NH_4^+ consumption remained constant in all three cultures (Table 2).

The effect of *N. winogradskyi* and heterotrophic bacteria on the growth rate of *Nitrosomonas* sp. Is79. The growth rate of *Nitrosomonas* sp. Is79 was determined in artificially designed co-cultures containing combinations of the isolated heterotrophic bacteria or *N. winogradskyi* over the course of 3 growth cycles (i.e., 2 culture transfers). The growth rate of *Nitrosomonas* sp. Is79 significantly increased in the presence of *N. winogradskyi* or heterotrophic isolates (Fig. 2). The growth rate of *Nitrosomonas* sp. Is79 co-cultured with all 10 heterotrophic isolates plus *N. winogradskyi* was comparable to the growth rate of *Nitrosomonas* sp. Is79 in the enrichment culture G5-7 (Fig. 2). In addition, the growth rate of *Nitrosomonas* sp. Is79 as part of the enrichment culture G5-7 was significantly higher than the growth rate of *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi* or any of the heterotrophic isolates (Fig. 2). The growth of *Nitrosomonas* sp. Is79 in the AOB-MS medium with R2A was not significantly different from its growth in AOB-MS medium (Fig. 2).

Chemostat growth. *Nitrosomonas* sp. Is79 was grown with NH_4^+ as the sole energy source and growth-limiting substrate as (i) a pure culture, (ii) in co-culture with *N. winogradskyi*, and (iii) as part of the enrichment culture G5-7 in continuous cultures. Consumption of NH_4^+ started 2 to 4 days after inoculation, the pumps for media addition and waste removal were started 5 to 10 days after inoculation, and all chemostats were run as continuous cultures for 3 volume changes (12 days) after reaching steady state (see Fig. S1 in the supplemental material). A temporary accumulation of NO_2^- was observed in the enrichment culture G5-7 and in the co-culture with *N. winogradskyi* (see Fig. S1). During steady-state growth, the concentration of the growth-limiting substrate, NH_4^+, in the chemostats was higher in the pure culture of *Nitrosomonas* sp. Is79 (115.94 μM) than in the co-culture of *Nitrosomonas* sp. Is79 with *N. winogradskyi* (2.54 to 6.35 μM) or the enrichment culture G5-7 (0.92 to 1.88 μM) (see Table S3).

Proteomics. SDS-PAGE of whole-cell extracts from the chemostat cultures showed different protein-banding patterns (see Fig. S2 in the supplemental material). However, direct inferences about the *Nitrosomonas* sp. Is79 proteome could not be made, because each culture contained a different bacterial community. Isobaric tagging using iTRAQ reagents followed by LC-MS/MS was used to determine the proteome of *Nitrosomonas* sp. Is79 in the chemostat cultures. Relative protein abundance ratios were based on the abundance of detected iTRAQ tags for each protein in each sample. The proteomic analysis presented here focused solely on *Nitrosomonas* sp. Is79 proteins that significantly changed in abundance (positive or negative change by a factor of >1.5; $P <$ 0.01) when co-cultured with *N. winogradskyi* or as part of the enrichment culture G5-7, relative to the pure culture. A full list of detected proteins, their change in abundance, and their calculated significances can be found in Tables S4 and S5 in the supplemental material. Due to technical problems, only one *Nitrosomonas* sp. Is79 chemostat culture was analyzed and used for comparison to the biological duplicates of *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi* (Set1) and G5-7 (Set2) chemostat cultures. Since accurate relative quantitation is predicated on uniform labeling of the primary amines with the iTRAQ reagents, the overall iTRAQ label efficiency was captured for all peptides detected in each comparative set. For Set1, 100% of 10,317 peptides terminating in lysine and 95.3% of 16,523 peptides at the amino terminus were labeled with an iTRAQ tag. For Set2, 100% of 9,684 peptides terminating in lysine and 95.6% of 15,318 peptides at the amino terminus were labeled with an iTRAQ tag. Thus, collectively, a labeling efficiency above 95% allows for excellent relative quantitation measurement.

The influence of *N. winogradskyi* on the proteome of *Nitrosomonas* sp. Is79. The levels of 55 *Nitrosomonas* sp. Is79 proteins

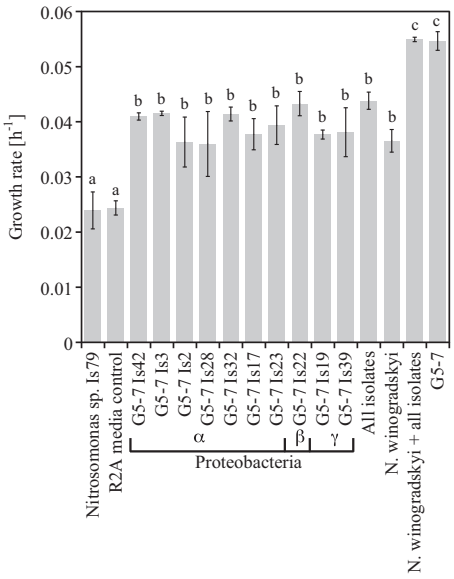

FIG 2 Growth (h^{-1}) of *Nitrosomonas* sp. Is79 in co-culture with different combinations of *N. winogradskyi* and heterotrophic bacteria isolated from the enrichment culture G5-7 during the third growth cycle (mean ± standard deviation; $n = 3$). *Nitrosomonas* sp. Is79 was combined with all of the heterotrophic isolates and *N. winogradskyi* individually in co-cultures and with a mixed culture containing all heterotrophs with and without *N. winogradskyi* (All isolates and *N. winogradskyi* + all isolates). *Nitrosomonas* sp. Is79 was also tested as part of the enrichment culture G5-7. R2A medium was added in place of heterotrophic bacteria as a control. Letters indicate significant differences between *Nitrosomonas* sp. Is79, the enrichment culture G5-7, and each particular treatment determined by one-way ANOVA followed by Tukey test ($P < 0.05$).

significantly changed in abundance by a factor of more than 1.5 ($P < 0.01$) when co-cultured with *N. winogradskyi* (Table 3; see also Table S4 in the supplemental material). Of these 55 proteins, 42 had an assigned function, while 13 were hypothetical proteins (Table 3; see also Table S4). In total, 24 proteins increased and 31 decreased in abundance. Many of these proteins had cellular functions related to energy generation, oxidative stress response, nutrient-binding, macromolecule degradation, or carbon metabolism (Table 3). The ATP synthase F_1 complex proteins (subunits α, β, γ, and δ) significantly decreased in abundance, while the F_1 subunit ε and the F_o subunit β also decreased but had *P* values above 0.01 (Table 3; see also Table S4). Proteins related to the cellular oxidative stress response changed in a site-specific manner. The cytoplasmic proteins superoxide dismutase, rubrerythrin, thiol peroxidase, and two chaperonins decreased in abundance, while several periplasmic di-heme cytochrome peroxidases increased in abundance (Table 3). The abundance of proteins involved in macromolecule degradation also changed in a site-specific manner. The cytoplasmic proteins aminopeptidase A and polyribonucleotide nucleotidyltransferase decreased in abundance, while a periplasmic lipase and RNase increased in abundance (Table 3). Nutrient-binding proteins for sulfate (periplasmic sulfate-binding protein), copper (CopC), and iron (extracellular solute-binding protein family 1) all increased in abundance (Table 3). Proteins related to central carbon metabolism, such as phosphoglycerate kinase, ribulose-phosphate 3-epimerase, and the flavoprotein subunit of the succinate dehydrogenase complex, decreased in abundance in the presence of *N. winogradskyi* (Table 3). Interestingly the relative abundance of the copper-containing nitrite reductase NirK increased when *Nitrosomonas* sp. Is79 was co-cultured with *N. winogradskyi* (Table 3).

The influence of the G5-7 community on the proteome of *Nitrosomonas* sp. Is79. Seventy *Nitrosomonas* sp. Is79 proteins significantly changed in abundance, by a factor of >1.5 ($P < 0.01$), in the enrichment culture G5-7 relative to their abundance in the pure culture of *Nitrosomonas* sp. Is79 (Table 3; see also Table S5 in the supplemental material). The abundance of 23 proteins increased, and the abundance of 47 decreased; out of these 70 proteins, 55 had an annotated function. Most changes in the *Nitrosomonas* sp. Is79 proteome observed when *Nitrosomonas* sp. Is79 was co-cultured with *N. winogradskyi* were also observed in the enrichment culture G5-7. Similar decreases of ATP synthase F_1 complex proteins and increases in nutrient-binding proteins were detected. In addition, proteins involved in the cellular oxidative stress response and macromolecule degradation decreased in abundance in the cytoplasm, but increased in the periplasm. In contrast to being co-cultured with only *N. winogradskyi*, several *Nitrosomonas* sp. Is79 proteins involved in ammonia oxidation increased in abundance in the enrichment culture G5-7: ammonia monooxygenase subunits A and B, open reading frame 5 (ORF5), hydroxylamine dehydrogenase, cytochrome P460 and cytochrome c_{554} (Table 3; see also Table S5).

The influence of heterotrophic bacteria on the proteome of *Nitrosomonas* sp. Is79. The effect of the heterotrophic community in the enrichment culture G5-7 was determined indirectly. If significant changes (positive or negative change by a factor of >1.5; $P < 0.01$) were observed in the presence of *N. winogradskyi* and in the enrichment culture G5-7, these changes were assumed to be due to the presence of the NOB *N. winogradskyi*. Significant changes (positive or negative change by a factor of >1.5; $P < 0.01$) only detected in the presence of the heterotrophic bacteria (G5-7) were assigned to be the effect of the heterotrophic bacteria (Table 3). One major effect of the presence of the heterotrophic community was the increase of proteins related to the ammonia oxidation pathway. In the presence of the heterotrophic community, the abundance of proteins related to carbon metabolism decreased, i.e., triosephosphate isomerase, enolase, phosphofructokinase, succinyl coenzyme A (succinyl-CoA) ligase subunit β, transketolase and the ribulose bisphosphate carboxylase oxygenase (RuBisCO) large- and small-chain subunits (Table 3). In addition, several proteins involved in amino acid synthesis and metabolism, such as glycine dehydrogenase subunits 1 and 2, threonine synthase, glycine hydroxymethyltransferase, and ATP phosphoribosyltransferase, were less abundant in the presence of heterotrophic bacteria (Table 3).

DISCUSSION

Individually as well as together, *N. winogradskyi* and the heterotrophic community of the enrichment culture G5-7 positively affected the growth of *Nitrosomonas* sp. Is79 (Fig. 1 and 2 and Table 2; see also Fig. S1 and Table S3 in the supplemental material). When co-cultured with *N. winogradskyi* or as part of the enrichment culture G5-7, *Nitrosomonas* sp. Is79 grew at a lower steady-state NH_4^+ concentration in continuous culture and at a significantly higher growth rate in batch culture (Fig. 1 and 2 and Table 2; see also Fig. S1 and Table S3). We used this collection of cultures to investigate the mechanisms underlying the positive influence of NOB and heterotrophic bacteria on the growth of *Nitrosomonas* sp. Is79. iTRAQ proteomics was used to determine the effect of *N. winogradskyi* and the heterotrophic community of G5-7 on the proteome of *Nitrosomonas* sp. Is79 when grown in continuous cultures (Table 3; Fig. 3).

Effects of *N. winogradskyi*. In AOB-NOB co-cultures, NO_2^- is removed by NOB, resulting in a relief of NO_2^--induced toxic effects and stress on AOB (3, 29, 63). Direct comparison of the proteome of *Nitrosomonas* sp. Is79 when grown in pure culture and when co-cultured with *N. winogradskyi* was used to determine possible mechanisms contributing to the positive influence of *N. winogradskyi* on the growth *Nitrosomonas* sp. Is79. Site-specific changes of several proteins related to oxidative stress were observed in the presence of *N. winogradskyi* (Table 3). The abundance of proteins responsible for the neutralization of reactive oxygen species significantly decreased in the cytoplasm and increased in the periplasm, while intracellular chaperonins also decreased (Table 3; Fig. 3). These proteome shifts indicate that the removal of NO_2^- from the growth environment of *Nitrosomonas* sp. Is79 resulted in a reduction of intracellular oxidative stress for *Nitrosomonas* sp. Is79. The reduced intracellular stress response very likely freed up energy in the form of proton motor force (PMF), ATP, or NADPH, as *Nitrosomonas* sp. Is79 would have less intracellular damage to repair. More energy could then be used for biomass production and growth. Oxidative stress relief has been shown to be the underlying mechanism for other microbial autotroph-heterotroph positive interactions, i.e., between the photoautotroph *Prochlorococcus* and the marine heterotroph *Alteromonas* sp. (64).

Nitrosomonas sp. Is79, like *N. europaea*, utilizes PMF to produce both ATP and NADPH (65). NADPH is generated through reverse electron flow, which makes the production of NADPH energetically more expensive for AOB than the production of ATP

TABLE 3 Significant changes[a] in relative abundance to *Nitrosomonas* sp. Is79 proteins in Is79 + NOB[b] or as part of the G5-7 enrichment culture compared to growth as pure culture[c]

gi no.	Locus tag (Nit79A3)	Description	Influence of: *N. winogradskyi* (Rep1/Rep2)	Influence of: G5-7 (Rep1/Rep2)	Influence of: Heterotrophic community of G5-7 (Rep1/Rep2)[d]
Energy generation and nitrogen metabolism					
gi|338804015	370	ATP synthase F_1 complex subunit delta	−1.50/−1.73		−
gi|338804016	371	ATP synthase F_1 complex subunit alpha	−2.55/−3.20	−5.35/−5.01	−
gi|338804017	372	ATP synthase F_1 complex subunit gamma	−1.65/−1.91	−2.05/−2.17	−
gi|338804018	373	ATP synthase F_1 complex subunit beta	−2.47/−2.84	−4.12/−3.98	−
gi|338805865	2335	Nitrite reductase, copper-containing (NirK)	+2.40/+3.54	+4.34/+3.00	−
gi|338806391	2882	Hypothetical protein: ORF5	−1.91/−2.04	−2.78/−2.64	−
gi|338806393	2884	Ammonia monooxygenase, subunit B		+1.99/+1.70	+
gi|338806394	2885	Ammonia monooxygenase, subunit A		+2.04/+1.93	+
gi|338806449	2940	Cytochrome c_{554}		+7.13/+3.58	+
gi|338806451	2942	Hydroxylamine dehydrogenase		+1.74/(+1.28)[e]	+
gi|338805203	1628	Cytochrome P460		+2.96/+2.46	+
Oxidative stress response					
gi|338803966	317	Alkyl hydroperoxide reductase/thiol specific antioxidant	(−1.42)/−1.50		−
gi|338804387	754	Di-heme cytochrome *c* peroxidase	(+1.35)/+1.89		−
gi|338804428	795	Di-heme cytochrome *c* peroxidase	+1.99/+2.99	+3.28/+2.32	−
gi|338805421	1860	Di-heme cytochrome *c* peroxidase	+1.55/+1.87		−
gi|338805484	1928	ATP-dependent Clp protease proteolytic subunit		−1.73/−1.69	+
gi|338805487	1931	60-kDa chaperonin (GroEL)	(−1.42)/−1.79	−1.82/−2.15	−
gi|338805488	1932	10-kDa chaperonin (GroES)	−1.59/−1.57	−1.85/−2.11	−
gi|338804827	1225	Thiol peroxidase	−1.75/−1.52	−2.19/−1.95	−
gi|338805541	1988	Manganese/iron superoxide dismutase	−1.74/−1.73	−2.29/−2.73	−
gi|338805570	2021	Cysteine desulfurase, SufS subfamily	(−1.49)/−1.80	−2.21/−2.18	−
gi|338806009	2479	Rubrerythrin	−1.80/−1.51	−2.00/−2.33	−
gi|338806646	3150	Ankyrin	+1.97/+1.75		−
gi|338805517	1961	Sigma E regulatory protein, MucB/RseB		+1.72/+1.85	+
Carbon metabolism					
gi|338804098	457	Phosphoglycerate kinase	−1.64/−1.78	−3.14/−2.89	−
gi|338805124	1544	Glyoxalase/bleomycin resistance protein/dioxygenase	(−1.47)/−1.74		−
gi|338805269	1697	Ribulose-phosphate 3-epimerase	−1.52/−1.77	−2.97/−2.98	−
gi|338806036	2508	6-phosphogluconate dehydrogenase	−2.36/−2.18		−
gi|338806059	2533	Succinate dehydrogenase, flavoprotein subunit	−1.59/−1.48		−
gi|338803769	104	Succinyl-CoA ligase (ADP-forming) subunit beta		−1.66/−1.67	+
gi|338803900	235	Phosphofructokinase		(−1.48)/−1.70	+
gi|338804096	455	Transketolase		−1.57/−1.83	+
gi|338805641	2094	Enolase		(−1.39)/−1.65	+
gi|338806444	2935	Triosephosphate isomerase		−1.61/−1.79	+
gi|338806728	3236	Ribulose bisphosphate carboxylase small chain		(−1.17)/−1.54	+
gi|338806727	3235	Ribulose bisphosphate carboxylase large chain		(−1.15)/−1.58	+
Nutrient binding					
gi|338804077	436	Hemolysin-type calcium-binding region	+3.29/+2.63	(+1.43)/+1.91	−
gi|338804618	997	Sulfate ABC transporter, periplasmic sulfate-binding protein	+1.50/+1.72	+2.09/+1.78	−
gi|338804785	1174	Extracellular solute-binding protein family 1	+2.28/+2.17	+2.22/+1.85	−
gi|338806154	2629	OmpA/MotB domain protein	−1.54/−1.51	−1.64/−1.63	−
gi|338807030	3552	Copper resistance protein CopC	+2.10/+1.91		−

(Continued on following page)

TABLE 3 (Continued)

gi no.	Locus tag (Nit79A3)	Description	Influence of: *N. winogradskyi* (Rep1/Rep2)	G5-7 (Rep1/Rep2)	Heterotrophic community of G5-7 (Rep1/Rep2)[d]
Macromolecule degradation					
gi|338805167	1591	Polyribonucleotide nucleotidyltransferase	(−1.32)/−1.64	−1.52/−1.57	−
gi|338805200	1625	RNase T2	+5.15/+3.58	+2.93/+2.83	−
gi|338805583	2034	Cytosol aminopeptidase	−1.63/−1.57		−
gi|338806285	2776	Lipase class 3	+2.18/+2.13	+3.12/+2.53	−
gi|338806518	3014	Alpha-glucan phosphorylase		−2.03/−1.79	+
gi|338806969	3490	Peptidase M4 thermolysin		(−1.29)/−1.73	+
Nitrogen assimilation and amino acid synthesis/metabolism					
gi|338804310	673	Aminotransferase class V	−1.69/−2.03	−2.57/−2.67	−
gi|338805895	2365	Glutamine synthetase, type I	(−1.40)/−1.54	−2.09/−2.21	−
gi|338806043	2515	Nitrogen regulatory protein P-II	−2.38/−2.11	−1.52/−1.52	−
gi|338806861	3379	Alanine dehydrogenase/PNT domain protein	(−1.35)/−1.51	−2.15/−2.01	−
gi|338803826	161	Glycine hydroxymethyltransferase		−1.60/−1.69	+
gi|338804474	841	Threonine synthase		−1.65/(−1.48)	+
gi|338804826	1224	Glycine dehydrogenase (decarboxylating) subunit 1		−1.99/−2.26	+
gi|338804828	1226	Glycine dehydrogenase (decarboxylating) subunit 2		−1.75/−1.93	+
gi|338805276	1704	Glutamate-1-semialdehyde 2,1-aminomutase		−1.50/−1.57	+
gi|338805542	1989	ATP phosphoribosyltransferase		−1.53/(−1.42)	+
gi|338806404	2895	2,3,4,5-tetrahydropyridine-2,6-dicarboxylate *N*-succinyltransferase		(−1.10)/−1.55	+
gi|338806806	3317	Glu/Leu/Phe/Val dehydrogenase		−1.52/−1.50	+
Replication, transcription, translation, and nucleotide biosynthesis					
gi|338804081	440	Integration host factor subunit beta	−1.83/(−1.25)		−
gi|338804209	572	Dihydroorotase, multifunctional complex type	−1.53/(−1.23)		−
gi|338804216	579	PpiC-type peptidyl-prolyl cis-trans isomerase	(+1.45)/+1.67	+1.72/+1.94	−
gi|338804452	819	50S ribosomal protein L7/L12	−1.93/−1.79	−2.32/−1.86	−
gi|338804691	1073	Peptidyl-prolyl *cis-trans* isomerase cyclophilin type	+2.34/+1.56		−
gi|338805468	1912	*S*-adenosylmethionine synthase	(−1.43)/−1.66	−2.19/−2.42	−
gi|338803669	2	DNA polymerase III, beta subunit		−1.62/−1.52	+
gi|338806332	2823	Translation elongation factor Tu		+2.14/+1.63	+
gi|338806665	3172	Single-strand binding protein		−1.67/−1.87	+
Miscellaneous					
gi|338806370	2861	Tetratricopeptide TPR_2 repeat-containing protein	+1.56/(+1.45)		−
gi|338804646	1025	Peptidoglycan-binding domain-containing protein		+2.79/+2.73	+
Bacteriophage related					
gi|338804625	1004	Phage tail sheath protein, putative		+2.07/+1.56	+
Hypothetical and unknown function					
gi|338804162	524	Hypothetical protein	+3.26/+2.88		−
gi|338804471	838	Hypothetical protein	+2.57/+3.81	+3.59/+2.96	−
gi|338804597	974	Hypothetical protein	−2.70/−1.67	(−1.25)/−1.68	−

(Continued on following page)

TABLE 3 (Continued)

gi no.	Locus tag (Nit79A3)	Description	Influence of: *N. winogradskyi* (Rep1/Rep2)	Influence of: G5-7 (Rep1/Rep2)	Influence of: Heterotrophic community of G5-7 (Rep1/Rep2)[d]
gi|338804892	1299	Hypothetical protein	−1.72/−1.58	−2.25/−1.91	−
gi|338805151	1573	Protein of unknown function	+1.87/+2.02		−
gi|338805427	1867	Hypothetical protein	+1.75/+2.01	+3.14/+2.13	−
gi|338805428	1868	Hypothetical protein	+1.64/+1.70	+2.17/+1.54	−
gi|338805889	2359	Hypothetical protein	+1.85/+2.49		−
gi|338805974	2444	Hypothetical protein	+2.21/+1.84	+2.10/+1.59	−
gi|338806490	2984	Hypothetical protein	+1.57/(+1.44)		−
gi|338806603	3107	Hypothetical protein	+1.96/+1.53		−
gi|338806604	3108	Hypothetical protein	+2.26/(+1.38)		−
gi|338806650	3154	Hypothetical protein	−1.52/−1.65	−2.00/−2.54	−
gi|338804639	1018	Hypothetical protein		+1.72/+1.61	+
gi|338805185	1609	Hypothetical protein		(−1.49)/−1.89	+
gi|338805805	2265	Hypothetical protein		+1.79/(+1.41)	+
gi|338806101	2576	Hypothetical protein		(−1.47)/−1.77	+
gi|338806350	2841	Hypothetical protein		−1.69/−1.79	+
gi|338806966	3487	Hypothetical protein		−2.31/−2.69	+

[a] Significance, >1.5-fold positive or negative change; $P < 0.01$.
[b] Is79 + NOB, *Nitrosomonas* sp. Is79 proteins when in co-culture with *N. winogradskyi*.
[c] All co-cultures (*N. winogradskyi* Reps 1 and 2, and G5-7 Reps 1 and 2) were compared to one control of *Nitrosomonas* sp. Is79. Positive (+) or negative (−) notations for the *N. winogradskyi* and G5-7 replicates (Rep1/Rep2) indicate increased or decreased absolute abundance, respectively.
[d] Indirect measurements on the effect of the heterotrophic community are indicated by + or − in the last column (see the text for details).
[e] Relative protein abundance changes reported that are less than a >1.5-fold positive or negative change are shown in parentheses.

(65). The abundance of several protein components of the F_1-ATP synthase were significantly lower when *Nitrosomonas* sp. Is79 was co-cultured with *N. winogradskyi* (Table 3; Fig. 3), indicating that *Nitrosomonas* sp. Is79 reduced ATP production in the presence of *N. winogradskyi*. This suggests that *Nitrosomonas* sp. Is79 co-cultured with *N. winogradskyi* could divert a larger portion of PMF produced by ammonia oxidation toward NADPH production. The increased generation of reducing power at the expense of lower ATP production would favor biosynthetic pathways, such as carbon fixation. This contrasts with a study focusing on the transcriptome of *N. europaea* in the presence of *N. winogradskyi*, which found *N. europaea* ATP synthase subunit transcripts upregulated in co-cultures with *N. winogradskyi* (29). These differences could be due to AOB exhibiting species-specific responses to NOB, different tolerances to oxidative and nitrosative stress, or dynamic transcriptional, translational, and posttranslational levels of control (40).

The abundance of the nitrite reductase NirK increased when *Nitrosomonas* sp. Is79 was co-cultured with *N. winogradskyi* (Table 3; Fig. 3). However, *nirK* in *N. europaea* has been shown to be induced by NO_2^- accumulation and downregulated in the presence of *N. winogradskyi* (29, 63). *Nitrosomonas* sp. Is79 lacks most of the nitrogen oxide metabolism genetic inventory present in *N. europaea*, including the rest of the *nir* operon; therefore, it is possible that NirK plays different roles in *Nitrosomonas* sp. Is79 and *N. europaea* (20, 66). During ammonia oxidation, NirK is hypothesized to act as an electron sink and prevent the buildup of the toxic intermediate hydroxylamine in AOB (67, 68). NirK has been shown to be essential for efficient ammonia oxidation in *N. europaea*, with ammonia oxidation rates decreasing in *nirK* mutants compared to the rate in the wild type (68). Since the NO_2^- concentration in the chemostats decreased from 4,653 μM in the absence of *N. winogradskyi* to below 15 μM when *N. winogradskyi* was present (see Table S3 in the supplemental material), our results suggest that NirK in *Nitrosomonas* sp. Is79 might be associated with more efficient ammonia oxidation rather than acting in response to NO_2^-. Future work is required to decipher the exact and possibly multiple roles of NirK in different AOB.

In summary, the shifts in the proteome of *Nitrosomonas* sp. Is79 in the presence of *N. winogradskyi* were very likely caused by the reduction of the NO_2^- stress and resulted in a decrease of energy spent in stress response, more energy efficient ammonia oxidation, and a subsequently higher growth rate (Table 2; Fig. 2 and 3). This conclusion is supported by the lower K_s and the higher growth rate of *Nitrosomonas* sp. Is79 when co-cultured with *N. winogradskyi* in batch culture (Table 2; Fig. 2).

Effects of heterotrophic bacteria. The heterotrophic community in the enrichment culture G5-7 consists of *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Bacteriodetes* (see Table S2 in the supplemental material). Members of these phylogenetic groups have previously been found in AOB and cyanobacterial enrichment cultures (26, 27, 69, 70). The addition of heterotrophic isolates as single isolates or together as a community resulted in enhanced growth rates of *Nitrosomonas* sp. Is79 (Fig. 1 and 2). Positive effects of heterotrophic bacteria on the growth and metabolism of autotrophic bacteria have been previously observed in methanotrophic bacteria (71) and cyanobacteria (64, 69, 70, 72). The underlying mechanisms of these interactions appear broad and are often not limited to distinct phylogenetic groups. Heterotrophic bacteria are hypothesized to provide a common cellular metabolite, eliminate toxic compounds, or reduce oxidative stress on autotrophs (70–74).

When *Nitrosomonas* sp. Is79 was grown as part of the enrichment culture G5-7, several proteins directly related to the ammo-

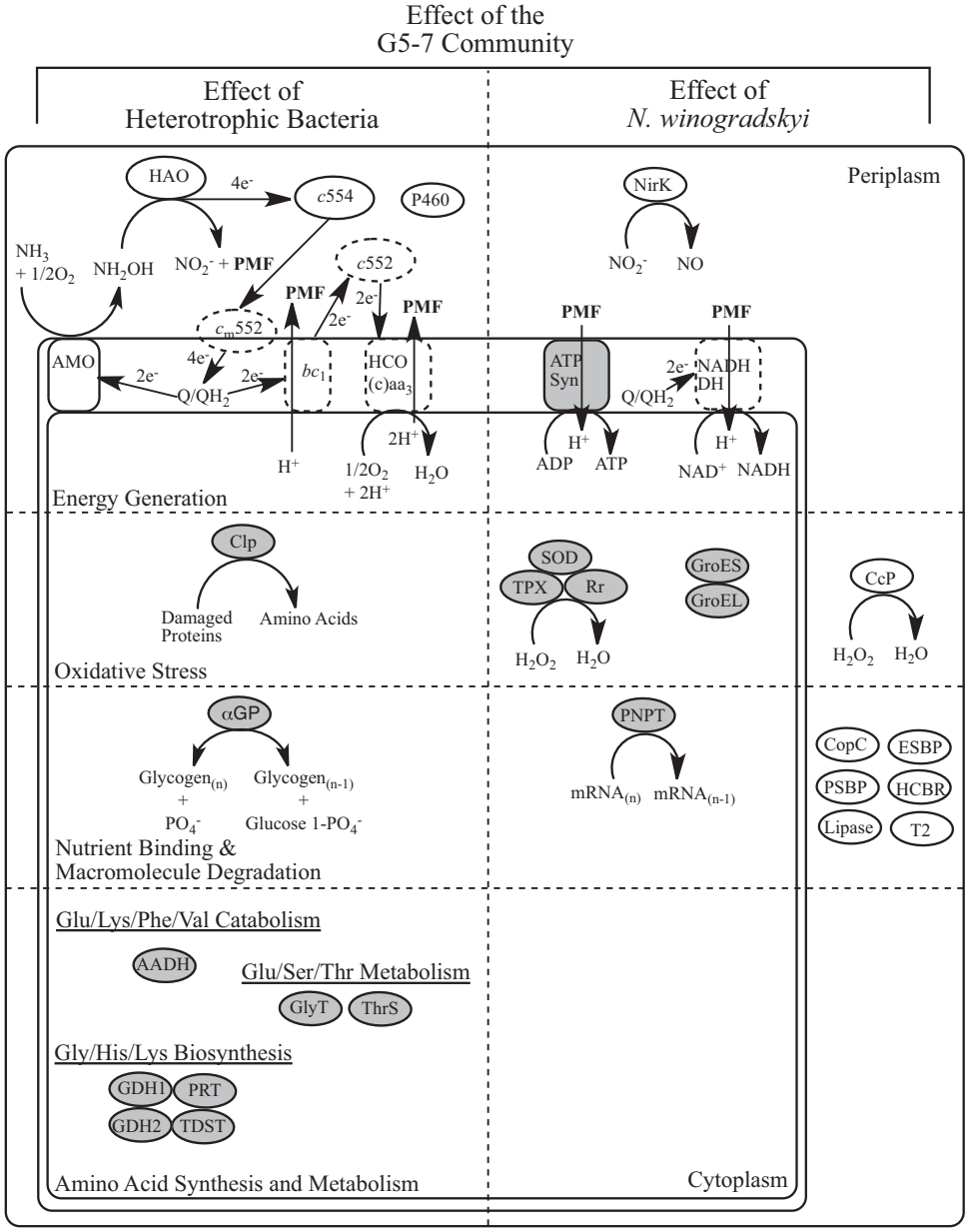

FIG 3 Model of the effect of heterotrophic bacteria (left) and *N. winogradskyi* (right) on the proteome of *Nitrosomonas* sp. Is79. Select proteins that increased (white) or decreased (gray) in abundance are shown. Proteins with dashed lines are shown for illustrative purposes only and were not significantly differentially regulated. AMO, ammonia monooxygenase; HAO, hydroxylamine dehydrogenase; c554, cytochrome c_{554}; c_m552, cytochrome c_{m552}; Q/QH_2, ubiquinone-ubiquinol pool; bc_1, cytochrome bc_1 (complex III); c552, cytochrome c_{552}; HCO (c)aa_3, cytochrome (*c*)aa_3; ATP Syn, ATP synthase; NADH DH, NADH dehydrogenase; PMF, proton-motive force; P460, cytochrome P460; GDH1, glycine dehydrogenase subunit 1; GDH2, glycine dehydrogenase subunit 2; ThrS, threonine synthase; GlyT, glycine hydroxymethyltransferase; GroEL, 60-kDa chaperonin; GroES 10-kDa chaperonin; TDST, 2,3,4,5-tetrahydropyridine-2,6-dicarboxylate *N*-succinyltransferase; AADH, Glu/Leu/Phe/Val dehydrogenase; PRT, ATP phosphoribosyltransferase; SOD, superoxide dismutase; Rr, rubrerythrin; NirK, nitrite reductase; TPX, thiol peroxidase; Ahp, alkyl hydroperoxide reductase; CcP, di-heme cytochrome *c* peroxidase; Clp, Clp protease subunit; αGP, α-glucan phosphorylase; PNPT, polyribonucleotide nucleotidyltransferase; CA, cytosol aminopeptidase; CopC, copper resistance protein; ESBP, extracellular solute-binding protein family 1; PSBP, sulfate ABC transporter periplasmic binding protein; HCBR, hemolysin-type calcium-binding region; Lipase, lipase class 3; T2, RNase T2.

nia oxidation pathway were significantly more abundant relative to their presence in the *Nitrosomonas* sp. Is79 and *N. winogradskyi* co-culture (Table 3). *Nitrosomonas* sp. Is79 relies on ammonia oxidation for energy generation, and a higher abundance of proteins involved in ammonia oxidation could result in more energy production and faster growth (Fig. 3). The increased growth rate and ability of *Nitrosomonas* sp. Is79 in the enrichment culture G5-7 to grow at a lower steady-state NH_4^+ concentration (Fig. 2; see also Table S3 and Fig. S1 in the supplemental material) support this conclusion.

In addition, 6 proteins related to amino acid synthesis and metabolism significantly decreased in abundance when *Nitro-*

somonas sp. Is79 was grown as part of the enrichment culture G5-7 (Table 3; Fig. 3). This suggests that, when grown in co-culture with other heterotrophic bacteria, *Nitrosomonas* sp. Is79 might down-regulate specific amino acid biosynthetic pathways, because they receive exogenous amino acid(s) or amino acid metabolite(s) from the heterotrophic community (Fig. 3). In support of this idea, certain amino acids have previously been shown to enhance the growth rate of *N. europaea* (34, 35, 75) and *Nitrosomonas* sp. Is79 has two annotated amino acid transporters in its genome (20). The positive interaction between *Nitrosomonas* sp. Is79 and the diverse group of heterotrophic bacteria (Table 1; see also Table S2 in the supplemental material) could therefore involve the exchange of amino acids and/or amino acid metabolites. Amino acid exchange has been shown to facilitate bacterial interactions between *Porphyromonas gingivalis* and *Treponema denticola* (76).

The effect of *N. winogradskyi* and heterotrophic bacteria combined. The shifts in the proteome of *Nitrosomonas* sp. Is79 grown as a part of G5-7 are a combination of the effects of both *N. winogradskyi* and heterotrophic bacteria (Table 3; Fig. 3). The combination of positive interactions in the enrichment culture G5-7 resulted in the lowest K_s (Table 2), the lowest steady-state NH_4^+ concentration in continuous culture (0.75 to 1.22 μM) (see Table S2 in the supplemental material) and the highest growth rate in batch culture (Fig. 2) of *Nitrosomonas* sp. Is79. The increased abundance of proteins involved in energy generation and the decreased abundance of ATP synthase proteins provides insight into how interactions with both *N. winogradskyi* and heterotrophic bacteria had a positive effect on the growth of *Nitrosomonas* sp. Is79 (Fig. 3). In summary, *Nitrosomonas* sp. Is79 positively interacted with both *N. winogradskyi* and heterotrophic bacteria individually and in defined mixed cultures, resulting in less stress and more energy for the AOB (Fig. 3). This type of controlled community culture experiment begins to bridge the gap between pure culture physiologic experiments and natural environmental microbial community studies.

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